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Enhancement of berberine production by spermidine in *Thalictrum minus* cell suspension cultures

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ABSTRACTS

Berberine production in cell suspension cultures of *Thalictrum minus* L. var. *hypoleucum* Miq. was significantly enhanced by administration of spermidine, whereas other polyamines such as cadaverine, putrescine and spermine were ineffective. The results of experiments indicated that spermidine causes an increase of ethylene generation which is closely associated with activation of berberine biosynthesis.

Keywords - *Thalictrum minus*, Ranunculaceae, cell suspension culture, ethylene, polyamines, spermidine, polyphenolics.

INTRODUCTION

We reported earlier that the administration of ethylene to cell suspension cultures of *Thalictrum minus* L. activated berberine biosynthesis in the early stage of culture, but it also enhanced cell browning due to the formation of bound polyphenols in the late stage of culture (Kobayashi et al. 1991). Thus, a continuous exposure of cells to ethylene at a high level of concentration over the entire culture period is not advisable to increase the final yield of berberine. Browning may be overcome by regulating the intracellular level of ethylene depending on the age or physiological condition of the cells, but it is technically difficult to adjust the concentration of ethylene to a desirable level time after time by adding either Ethrel (2-chloroethylphosphonic acid) , an ethylene generator (Kobayashi et al. 1990) or AVG (aminoethoxy-vinylglycine) , an inhibitor of ethylene biogenesis (Kobayashi et al. 1991), throughout the culture period.

As an alternative we made an attempt to control cell browning by treating cell cultures with polyamines, such as putrescine, cadaverine, spermidine, and spermine, which are known to be involved in various processes of plant growth and development (Phillip and Russell 1989; Slocum et al. 1984; Smith 1985). Furthermore, it has been reported that polyamines inhibit ethylene formation in various plant tissues (Apelbaum et al. 1981; Suttle 1981) as the antagonist of ethylene (Even-Chen et al. 1982). This paper reports the effects of polyamines on berberine production and cell browning in *T. minus* cell suspension cultures.

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MATERIALS AND METHODS

Chemicals. Putrescine-2HCl, cadaverine-2HCl, spermidine-3HCl, spermine-4HCl and AVG were purchased from Sigma.

Cell suspension cultures. Cell suspension cultures of *Thalictrum minus* L. var. *hypoleucum* Miq. (Nakagawa et al. 1984) were cultivated in growth medium after Linsmaier and Skoog (1965) containing 1 μM 2,4-dichlorophenoxy-acetic acid by subculturing every two weeks. To examine the effects of polyamines on berberine production and cell browning, cells (1 g fresh wt) were inoculated into 30 ml of the production medium (LS medium containing 100 μM 1-naphthaleneacetic acid and 10 μM 6-benzylaminopurine) in 100 ml Erlenmeyer flasks. The suspension cultures were agitated on a reciprocal shaker (100 strokes / min) in the dark at 25°C.

Quantification of berberine. The quantitative analysis of berberine was carried out according to the method described previously (Nakagawa et al. 1984).

Ethylene determination. Ethylene was determined by GC as described elsewhere (Kobayashi et al. 1991). The identity of ethylene was confirmed by performing the reaction with mercury perchlorate (Warner and Leopold 1969).

Quantitative estimation of bound polyphenols. Fresh cells were refluxed in EtOH for 2 h under argon gas, thrice. The extracts were combined and centrifuged at 1000 x *g* for 5 min. The pellet was refluxed in 1 N NaOH for 16 h under argon gas, and the precipitate obtained by centrifugation as above was used for the determination of lignin-like compounds according to the method of Fukuda and Komamine (1982) with a slight modification.

Administration of polyamines and AVG. Polyamines and

AVG were applied as an aqueous solution (0.5 ml) through a membrane filter to culture medium (30 ml). An equivalent volume of deionized water was added to the control culture.

RESULTS AND DISCUSSION

Fig. 1 shows the time-course of berberine production and cell growth in *T. minus* cell suspension cultures cultivated in production medium. Berberine production began on day 9 and continued until day 23 when the yield was 10 mg / 30 ml of medium. The cells secreted practically all the berberine produced into the medium, as reported previously (Yamamoto et al. 1987). Effects of polyamines (0.5-10 mM) added to the production medium at day 3 on berberine production and cell growth in the early stage of culture (day 9) are shown in Fig. 2. Of the four polyamines tested, only spermidine was effective in promoting berberine production, which increased by over 400% at the concentration of 2 mM in comparison with the control after 9 days of treatment. Cell growth was not affected at 2 mM of spermidine. By contrast, the other polyamines (putrescine, cadaverine, and spermine) were more or less inhibitory to both berberine production and cell growth. The stimulatory effect of spermidine as well as the inhibitory effect of the other polyamines were comfirmed by repeated experiments. As shown in Fig. 3, the final yield of berberine in a long-term culture was also significantly higher in the spermidine-treated culture, although the relative increment was smaller than that in the early stage of culture.

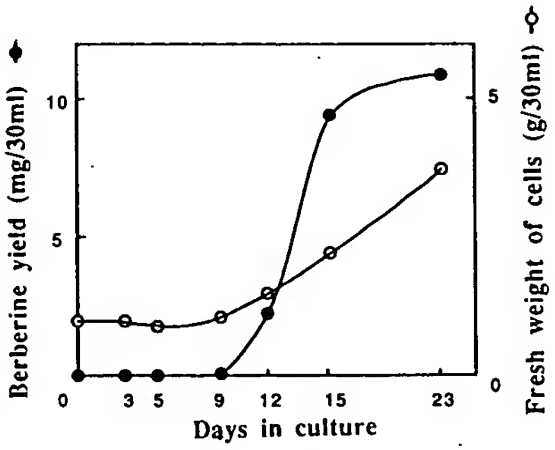

Fig. 1. Time-course of berberine production and cell growth in *T. minus* cell suspension cultures.

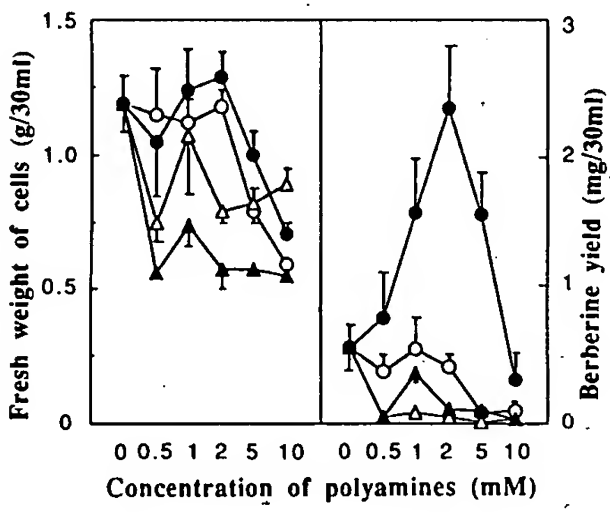

Fig. 2. Effects of polyamines on berberine production in *T. minus* cell suspension cultures in the early stage of culture. Polyamines were added to the culture medium on day 3 and cells were harvested after 9 days of incubation.

○:putrescine (Put), △:cadaverine (Cad), ● : spermidine (Spd) and ▲: spermine (Spm). Three replicates.

Fig. 4 showed the effect of polyamines on the generation of ethylene, an activating factor of berberine biosynthesis (Kobayashi et al. 1991). It has been reported for apple (Apelbaum et al. 1981) and *Tradescantia* (Suttle 1981) that polyamines reduced the amount of ethylene induced by senescence or by auxin. Similarly, putrescine, cadaverine, and spermine inhibited not only ethylene generation, but also berberine production in *T. minus* cell cultures. The administration of spermidine, on the contrary, significantly increased ethylene generation as well as berberine production. In addition, the promotion of berberine production by spermidine was strongly counteracted by AVG , which is known as a strong inhibitor of ethylene biosynthesis (Amrhein and Wenker 1979) (Fig. 5). These results suggest that spermidine enhances berberine production by promoting ethylene biosynthesis.

In spite of a significant increase in ethylene generation at day 9 that followed after the administration of spermidine (Fig. 4, 6), there was no significant

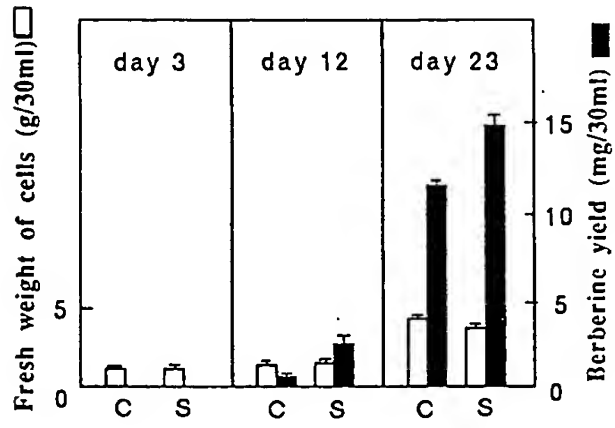

Fig. 3. Effects of spermidine on berberine production in *T. minus* cell suspension cultures during a long-term culture. Spermidine (2 mM) was added to the culture medium (30 ml) on day 3 and cells were harvested after 0, 9, and 20 days. C : control, S : spermidine. Three replicates.

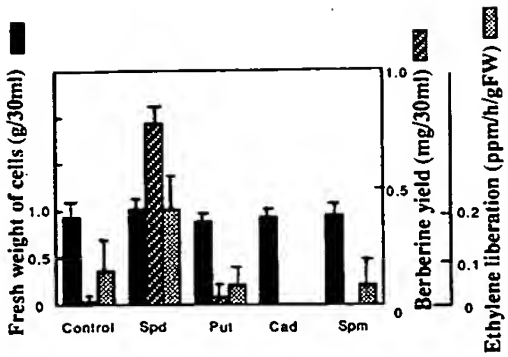

Fig. 4. Effects of polyamines on berberine production and ethylene generation in *T. minus* cell suspension cultures. Polyamines (2 mM) were added to the culture medium (30 ml) on day 3. Cells were harvested after incubation for 6 days. Three replicates.

increase in cell browning as well as in the formation of bound polyphenols (Fig. 7). These favorable effects on berberine production are presumably due to a moderate elevation of the ethylene level in the early stages of culture as well as to a retardation of cell senescence in the presence of a proper concentration of spermidine. At the present stage, we cannot conclude whether either a higher ethylene concentration or a longer time of exporsure of the cells to ethylene is important for increasing berberine production.

Although a number of studies have been made on physiological activities of polyamines in plants (Smith 1985), there are few reports concerning effects of polyamines on secondary metabolism. Muhitch and Fletcher (1985) showed that the addition of sucrose and spermidine to Paul's scarlet rose cell suspension cultures resulted in an increase of gallic acid and epicatechin. The effect of spermidine on ethylene generation in *T. minus* cell suspension cultures cannot be ascribed to a mere

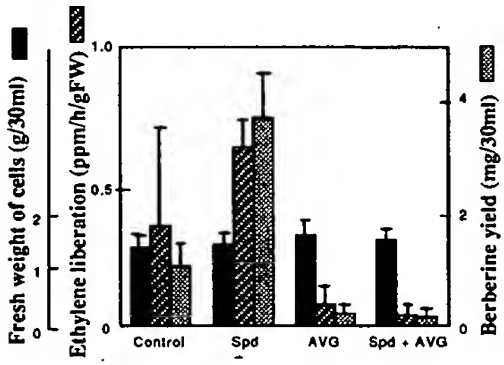

Fig. 5. Effect of AVG (an inhibitor of ethylene biosynthesis) on berberine production in *T. minus* cell suspension cultures. AVG (20 μg / ml) and spermidine (2 mM) were added to the culture medium (30 ml) on day 3 and cells were harvested after 6 days. Three replicates.

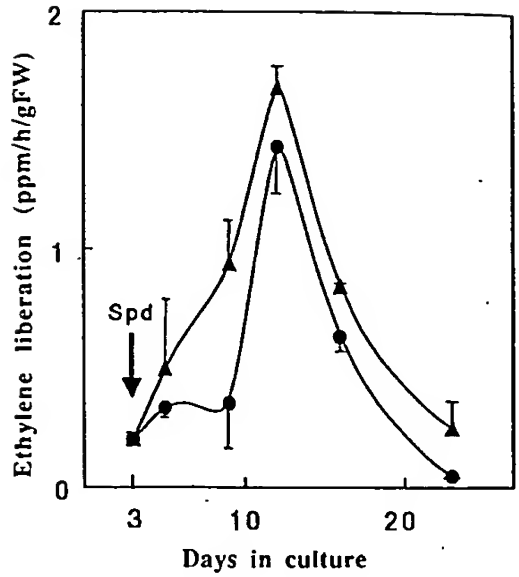

Fig. 6. Effect of spermidine on ethylene generation in *T. minus* cell suspension cultures. Spermidine (2 mM) was added to the culture medium (30 ml) on day 3. ●: control, ▲: 2 mM spermidine. Three replicates.

chemical stress that might be imposed on cells, since the addition of spermidine to the medium at a concentration between 0.5 and 5 mM neither impaired cell growth nor caused any noticeable cell browning. These cells showed a positive reaction in vital staining with neutral red and Evans blue (data not shown). Although the biochemical mechanism by which spermidine accelerates ethylene generation in *T. minus* cells remains to be elucidated,

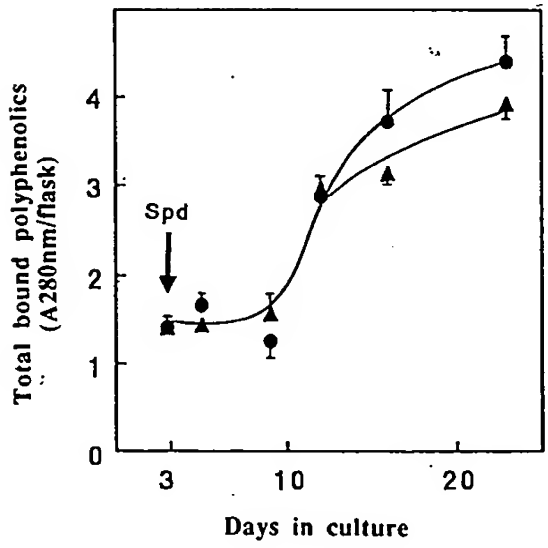

Fig. 7. Effect of spermidine on the formation of bound polyphenolics in *T. minus* cell suspension cultures. Spermidine (2 mM) was added to the medium (30 ml) on day 3. ●: control, ▲: 2 mM spermidine. Three replicates.

the use of spermidine may provide a condition favorable for the production of not only berberine but also of some other secondary metabolites.

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